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Turnover of organelles by autophagy in yeast

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Abstract

Efficient detection and removal of superfluous or damaged organelles are crucial to maintain cellular homeostasis and to assure cell survival. Growing evidence shows that organelles or parts of them can be removed by selective subtypes of otherwise unselective macro- and microautophagy. This requires both the adaptation of the core autophagic machinery and sophisticated mechanisms to recognize organelles destined for turnover. We review the current knowledge on autophagic removal of peroxisomes, mitochondria, ER and parts of the nucleus with an emphasis on yeasts as a model eukaryote.

Introduction

Autophagy encompasses all processes delivering cytosol and organelles to the lysosome for macromolecule turnover and recycling of building blocks. This definition includes non-vesicular cargo-delivery modes, such as “Chaperone Mediated Autophagy (CMA)”, and vesicular subtypes, such as macro- and microautophagy [1]. While microautophagy sequesters cytosol into lysosomal invaginations, macroautophagy requires the formation of double-membrane transport vesicles (autophagosomes) whose fusion with the lysosome releases autophagic bodies into the lysosome for degradation. The importance of autophagy during neurodegeneration, cancer, programmed cell death, ageing and removal of intracellular bacteria has drawn exponentially growing interest to these processes [1,2]. Starvation induced macroautophagy is generally considered an unselective process. In growing *S. cerevisiae* cells the cytosol-to-vacuole transport (Cvt) pathway, which morphologically and genetically resembles macroautophagy, selectively targets preaminopeptidase I (pApeI) and α -mannosidase to the vacuole [3]. Here we discuss the selective autophagic removal of superfluous or damaged organelles focusing on yeasts.

Macroautophagy in *S. cerevisiae*

Since the isolation of autophagy-mutants [4,5] 31 ATG- (AuTophagy) genes are known [6]. About half of the Atg-proteins form the core machinery, essential for the biogenesis of both autophagosomes and Cvt vesicles. Other Atg-proteins adapt this core machinery to the needs of autophagic subtypes.

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Autophagosome biogenesis starts with formation of a double-membrane structure termed the isolation membrane or phagophore, from a perivacuolar phagophore assembly site (PAS) (Fig. 1). After elongation of the isolation membrane, its edges are sealed. Remarkably, this fusion step does not require yeast t-SNAREs or the yeast NSF Sec18 [7,8] suggesting that Atg, and perhaps other, proteins mediate these functions. In *S. cerevisiae* the core Atg-proteins colocalize at the PAS, the site of autophagosome biogenesis [9–11], and form functional complexes, which assemble hierarchically.

The Atg1-kinase complex

The serine/threonine protein kinase, Atg1, and its regulatory factors Atg13, Atg17, Atg29 and Atg31 form a basic scaffold for PAS assembly during starvation [9–12]. Atg1 and Atg13 are core Atg proteins, while Atg17, Atg29 and Atg31 are dispensable for the Cvt pathway. In rich media, Atg13 is phosphorylated dependent on the rapamycin-sensitive TORC1. Upon induction of macroautophagy, Atg13 is dephosphorylated, and together with Atg17, Atg29, and Atg31, interacts with Atg1. This stimulates Atg1 kinase activity and modulates the autophagic response [3,10,13]. The kinase activity of Atg1 is essential for the Cvt pathway and macroautophagy, but dispensable for PAS assembly during starvation [3,9,10,13]. Probably, this kinase activity mediates dissociation of factors from the PAS.

Atg9 and its cycling system

The integral membrane protein, Atg9, is also recruited to the PAS early during its assembly. Atg9 additionally localizes to a peripheral compartment (PC) (at the ER or mitochondria) dependent on Atg1 (but not its kinase activity), Atg13, Atg2 and Atg18 [14,15]. The absence of Atg23 or Atg27 leads to Atg9 accumulation at the PC [3]. These findings led to a model wherein Atg9 cycles between its PC and the PAS [3] in vesicles that deliver membranes between these compartments. Since Atg9 is present at the PAS, but not on mature autophagosomes, this model postulates retrograde retrieval of Atg9 from the PAS. Atg23 and Atg27, proposed to act in Atg9 transport to the PAS, are specific for the Cvt pathway leading to the hypothesis that they only enhance Atg9 transport efficiency [3]. Self-association of Atg9 is also needed for efficient PAS targeting and its function during the Cvt pathway and macroautophagy [16].

The precise role of the PAS during biogenesis of autophagosomes is still poorly understood. In a model that probably best fits with the data, the core autophagic machinery first mediates assembly of the isolation membrane, then coordinates the incoming lipid flux and finally mediates sealing of the phagophore. Since components such as Atg9 are absent from mature autophagosomes, their retrograde retrieval must take place. In this model the PAS is a site where the autophagic machinery and the developing autophagosome converge.

The PtdIns3P-generating complex

In *S. cerevisiae* the sole PtdIns 3-kinase, Vps34, is present in two complexes. Complex I, consisting of Vps34, Vps15, Vps30/Atg6 and Atg14, localizes to the PAS and is required for autophagy. Complex II contains Vps38 instead of Atg14, acts in the vacuolar protein sorting pathway and localizes to endosomes and the vacuolar membrane [17]. A major function of PtdIns3P at the PAS is to recruit proteins such as Atg18. The *S. cerevisiae* genome contains Atg21 and Ygr223c, two Atg18 homologues. While Atg18 is a core Atg protein, Atg21 is Cvt-specific [18,19] and Ygr223c is needed for efficient micronucleophagy [20]. All three proteins bind PtdIns3P and PtdIns(3,5)P₂ [19,21,22]. Atg18 forms a complex with Atg2 and binds via PtdIns3P to the PAS [14,23] and to endosomes [23,24]. PtdIns(3,5)P₂ is not needed for macroautophagy or the Cvt pathway [21,25]. Accordingly, Atg18, recruited to the vacuolar membrane via PtdIns(3,5)P₂, carries out non-autophagic functions such as the retrograde

transport to the late endosome [21,26], a function which fits with its proposed role in Atg9 cycling.

The ubiquitin-like conjugation systems

Atg12 is covalently conjugated to Atg5 by a ubiquitin-related conjugation system consisting of Atg7 as an E1-like, and Atg10 as an E2-like, enzyme (Fig. 2) [13,27,28]. The Atg12-Atg5 conjugate then interacts with Atg16, whose self-interaction leads to oligomerization. This complex primarily decorates the outer membrane of the isolation membrane, but is absent from mature autophagosomes. This behaviour is reminiscent of coat complexes in vesicular trafficking in the secretory pathway, however a recent study suggests that the Atg16 level at the PAS is too low for such a function [29].

A second ubiquitin-like conjugation (Ublc) system using Atg7 as E1- and Atg3 as an E2-enzyme conjugates Atg8 to the membrane lipid phosphatidylethanolamine (PE) [13,28,30]. To allow lipidation, the carboxyterminus of Atg8 must first be processed by the cysteine protease Atg4. Efficient macroautophagy additionally requires Atg4 for deconjugation of Atg8, which is found at the isolation membrane and at autophagosomes. Despite the deconjugation activity of Atg4, part of Atg8 is enclosed into autophagosomes and finally degraded, opening a way to monitor autophagy. Atg8 mediates membrane tethering and hemifusion and its expression level correlates with the size of autophagosomes [31,32]. Atg8 hypothetically affects fusion of incoming vesicles with the isolation membrane during elongation and the final sealing of the phagophore.

The Atg12-Atg5 conjugate interacts with Atg3 and stimulates its activity similar to an E3 enzyme [33]. Work on a mammalian Atg16 homologue further suggests that it determines the cellular site of Atg8 lipidation [34].

Source of lipids for membrane expansion at the PAS

This is largely unknown. There are hints for an involvement of mitochondria [15], the trans-Golgi network and the endocytic system [35]. Additionally, the requirement of early secretory proteins for macroautophagy was shown, suggesting a role for the ER or Golgi [7,8].

Intravacuolar events

As a final step of macroautophagy the autophagic bodies are lysed inside the vacuole dependent on the lipase-like Atg15 and vacuolar proteinases [25].

The Cvt pathway

In the Cvt pathway, the cargo pApeI is important for PAS assembly and Cvt vesicle biogenesis (Fig. 1) [3]. pApeI assembles into a large complex, which recruits its cargo receptor Atg19. This receptor further interacts with Atg8 and the Cvt-specific protein Atg11, which functions as a basic scaffold in assembling the Cvt-specific PAS by interacting with itself and a multitude of proteins such as Atg1, Atg9 and Atg17 [3]. This scaffolding function of Atg11 in rich medium resembles the function of the Atg17 complex during starvation. The levels of Atg11 and Atg17 do not change during the switch from the Cvt pathway to macroautophagy [29]. However, the interaction of Atg1 and Atg17 is strengthened upon starvation [10], suggesting a role of protein complex formation in switching the PAS from the Cvt pathway to macroautophagy.

Pexophagy

Peroxisome number and size can change rapidly in response to environmental and/or physiological cues. In yeast, conditions in which peroxisome metabolism is required will stimulate the biogenesis of these organelles. Different inducers can induce different types of peroxisomes. For example, most yeasts metabolize oleate and this medium induces small non-clustered peroxisomes, but in addition, methylotrophic yeasts (e.g. *P. pastoris*, *H. polymorpha*) metabolize methanol, which induces giant, clustered peroxisomes. Independent of the type of peroxisomes induced, growth of these yeast cells in a carbon source where the peroxisome metabolism is not required will induce their degradation by a selective type of autophagy, referred to as pexophagy.

Two modes of pexophagy - **macropexophagy** and **micropexophagy** - analogous to macroautophagy and microautophagy, occur in *P. pastoris* and *H. polymorpha*, however pexophagy exists in all species [36–39] (Fig. 3). In macropexophagy, the isolation membrane engulfs one peroxisome at a time, forming a double-membrane vesicle called a pexophagosome, whose outer membrane fuses with the vacuolar membrane to deliver the pexophagic body and the peroxisome into the vacuolar lumen for degradation. In contrast, during micropexophagy, the vacuole membrane invaginates to engulf the peroxisome cluster. This membrane also protrudes along the peroxisome surface. This protrusion, denoted as the vacuolar sequestering membrane (VSM), often septates. In addition, a cup-shaped double-membrane structure, the micropexophagic membrane apparatus (MIPA), is formed on the peroxisome surface and mediates fusion between the VSM tips engulfing the peroxisomes [40].

The signaling events are not known but pexophagy can be induced by different media and the mode of pexophagy can vary [37,38,41]. Moreover, the same medium can induce different modes of pexophagy in different yeasts. Starvation can also induce pexophagy, together with autophagy, nevertheless in *P. pastoris*, pexophagy is a selective process and in the absence of the peroxisome receptor (Atg30), pexophagy does not occur [42]. In *P. pastoris*, ATP levels determine the mode of pexophagy; high ATP levels induce micropexophagy and lower levels induce macropexophagy [43]. The higher ATP demand of micropexophagy could be associated with an ATP-dependent actin rearrangement for invagination of the vacuolar membrane.

Macropexophagy involves a multi-step sequestration of peroxisomes from the cytosol into pexophagosomes. These steps include the induction of the pathway, cargo selection, nucleation of the isolation membrane, expansion of this membrane, pexophagosome completion, retrieval of autophagic machinery components, fusion of pexophagosomes with the vacuole, breakdown of the pexophagic body and peroxisomes, and recycling of the free amino acids and lipids to the cytosol. Because most of these steps are common to all autophagy-related pathways, it is not surprising that the same core Atg-proteins (see macroautophagy section) are shared. Pexophagy adapts the autophagic machinery to become selective. A few selective factors have been discovered: one is Atg11, also involved in the delivery of the cargo complex to the PAS and the organization of this structure in the Cvt pathway, and a similar role has been postulated for pexophagy [44]; another factor is Atg30, the peroxisome receptor for pexophagy and the final target of a signaling cascade resulting in a phosphorylation event that induces the pathway [42]. Atg30 localizes at peroxisome membrane, interacting with two peroxins, Pex3 and Pex14. During pexophagy it interacts with the autophagy machinery, through Atg17 and Atg11, the latter interaction being dependent on Atg30 phosphorylation. Pex3 and Pex14 also play an important role in pexophagy in *H. polymorpha* [39].

Most morphological studies on pexophagy have been done in *P. pastoris* and *H. polymorpha*, taking advantage of their giant methanol-induced peroxisome clusters. Because

of this giant cargo, the isolation membrane (pexophagosome and the MIPA) are larger and can be visualized distinctly from the other perivacuolar structures. As in *S. cerevisiae*, Atg8 localizes to these isolation membranes [40]. During peroxisome proliferation, the total Atg8 level increases, localizing mostly to the cytosol. However, upon a shift to pexophagy conditions, the Atg8 level remains the same but the lipidated Atg8 (Atg8-PE) levels double and its localization changes to a perivacuolar dot, the PAS, the site where the MIPA and pexophagosome will form. Most of the autophagic machinery localizes soon thereafter at the PAS, where proteins such as Atg5, Atg8, Atg9, Atg11, Atg16, Atg17, Atg24, Atg25, Atg26, Atg28 and Atg30 have been localized during peroxisome proliferation or pexophagy conditions [42,45–49]. The assembly of the MIPA from the PAS requires at least Atg1, Atg2, Atg3, Atg4, Atg7, Atg8, Atg9, Atg11, Atg26, Atg30 and Vps15 [39,42,47], (R. Mathewson, personal communication). In summary, MIPA and probably pexophagosome formation, require the complete set of core Atg-proteins and the pexophagy-specific factors Atg11, Atg26 and Atg30. In *H. polymorpha*, pexophagosome formation also requires Atg25, another protein that localized to this isolation membrane [46].

Atg26, a UDP-glucose:sterol glucosyltransferase, whose function has not been elucidated, is required exclusively for pexophagy of large, methanol-induced peroxisomes [41]. Atg26 binds phosphatidylinositol 4-phosphate (PtdIns4P), a lipid that is required for its localization at the MIPA and pexophagosome [49,50]. Therefore the formation of PtdIns4P by a phosphatidylinositol-4-OH kinase (Pik1) is essential for MIPA (and probably pexophagosome) formation, as well as for pexophagy in methylotrophic yeasts.

The signaling event that induces the formation of VSMs during micropexophagy is independent of Atg30 phosphorylation and even the presence of peroxisomes. Several mutants do not form the complete VSMs, such as $\Delta atg2$, $\Delta atg9$, $\Delta atg11$, $\Delta atg18$, $\Delta atg28$, $\Delta vac8$ and $\Delta vps15$ [39, 44,51–53]. Interestingly, Atg11 and Atg9 colocalize to perivacuolar dot-like structures (PVSs) distinct from the PAS, and located at the base of the VSMs [52]. This PVS is supposed to be important for VSM formation. Atg9 shuttles from a peripheral compartment (PC) [somewhere in the ER in *P. pastoris* or near mitochondria in *S. cerevisiae*] to the PVS, in an Atg11- and Vps15-dependent manner, but it is unclear if it goes directly or through the PAS [52]. The movement of Atg9 from the PVS to the VSM requires Atg2 [52], which shuttles from the cytosol to a dot-like structure juxtaposed to the vacuole, probably the PAS, and this trafficking requires the function of Atg1, Atg9, Atg18 and Vps15. It is possible that Atg2 and/or Atg9 trafficking contribute to deliver membrane to the PAS and/or PVS for the elongation of isolation membranes and/or VSMs, respectively. After the VSMs form, Atg11 localizes mainly on the VSM and this localization depends on Vac8, which is also localized at the VSM [44, 53]. Potentially, Atg11 at the VSM could interact with Atg30, to help peroxisome recognition by the VSMs.

Similar to the Cvt and autophagy pathways, the isolation membrane is absent in most of the autophagy mutants, suggesting the presence of retrograde pathways. A recent study suggests that the Ras-like small GTPase, Sar1, could play a role in recycling ER components from the pexophagosome/MIPA back to the ER, and this event could be a prerequisite for isolation membrane formation and maturation [54].

Finally, closure of the isolation membrane proceeds via the fusion of the MIPA with the VSMs, or by fusion of the pexophagosome with the vacuole membrane, for micropexophagy and macropexophagy, respectively. These fusion events require Atg24 [45], a protein that binds phosphatidylinositol 3-phosphate (PtdIns3P) and which accumulates at the vertex ring of the contact area between: (1) septated vacuoles, (2) the MIPA and tips of the VSMs, and, (3) the pexophagosome and vacuole membrane. Some Atg24 localizes close to or at the PVS, but no colocalization data are available. Vam7, a component of a t-SNARE complex, is also involved

in a late stage of pexophagy, probably in the fusion between the pexophagosome/MIPA and the vacuolar membranes. Vac8 is required for vacuolar homotypic fusion and it has been suggested that this function is also required for a late event of micropexophagy [53].

Micronucleophagy (Piecemeal microautophagy of the nucleus, PMN)

In *S. cerevisiae* the interaction of the ER membrane protein, Nvj1, and vacuolar Vac8 generates contacts between the nuclear envelope/ER and the vacuole. Upon starvation, in a process termed micronucleophagy or PMN, these junctions bulge into invaginations of the vacuole (Fig. 4). Subsequent fission of the nuclear ER-membranes and fusion of the vacuolar membrane then releases vesicles containing nuclear material into the vacuole for degradation [55]. Consequently, the PMN-vesicles are limited by three membranes, the outermost derived from the vacuole, the other two from the nuclear ER.

Nvj1 further recruits the enoyl-CoA reductase Tsc13 and Osh1, a homologue of a mammalian oxysterol-binding protein, to the nucleus-vacuole junctions [56]. Following the breakdown of these markers therefore permits monitoring of micronucleophagy, which requires, besides the core Atg-proteins, a set of subtype-specific Atg proteins, including Atg11 and Atg17 [20]. Efficient micronucleophagy also depends on the Atg18 homologue, Ygr223c [24]. Typical components of the homotypic vacuole fusion machinery are not needed for micronucleophagy [20]. Based on this and electron microscopic data, it has been speculated that the Atg-machinery might mediate the formation of the vacuolar protrusions and their final fusion - functions which are similar to those used during micropexophagy.

Mitophagy

Deterioration of mitochondrial function might lead to the production of excess reactive oxygen species or the untimely release of pro-apoptotic proteins causing cell death. Accordingly, as a quality control system, the controlled removal of mitochondria via autophagy is expected. Indeed, in autophagy-deficient *S. cerevisiae* cells, reduced mitochondrial functionality was detected, compatible with an accumulation of damaged mitochondria [57]. On the other hand, the lack of a mitochondrial membrane potential ($\Delta\Psi$) [58] and osmotic swelling caused by the depletion of Mdm38 [59] induced autophagic degradation of mitochondria. Also the lack of mitochondrial ATP-dependent metalloproteinase, Yme1, on non-fermentable carbon sources increased vacuolar degradation of mitochondria. Remarkably, these conditions did not increase vacuolar turnover of cytosol indicating selective mitophagy [60].

So far in *S. cerevisiae* the highest mitochondrial turnover was not measured by compromising mitochondrial function, but after shifting wild-type cells from a medium containing the non-fermentable carbon source, lactate, to nitrogen-free glucose-medium [61]. Under these conditions electron microscopy suggests the occurrence of two distinct types of micromitophagy. In subtype I, mitochondria are engulfed by vacuolar indentations together with cytosolic material, while in the variant II only mitochondria are sequestered [62]. Both mitochondrial uptake processes depend on almost the complete set of Atg-proteins, including the Cvt-specific, Atg11, and the macroautophagy-specific Atg17 and Atg29, but not on the pApeI-receptor, Atg19 [61,62]. The mitochondrial outer membrane protein, Uth1, is required only for micromitophagy subtype II [62], while the mitochondrial protein phosphatase homologue, Aup1, is generally implicated in mitophagy [63].

Mitochondria dynamically undergo fission and fusion events. Inhibition/deletion of the dynamin-like GTPase Drp1 (in yeast Dnm1) interfered with mitophagy in both mammalian and *S. cerevisiae* cells, showing the importance of mitochondrial fission for mitophagy [59, 64]. In mammalian cells the occurrence of a macromitophagic process was suggested, which

might be due to the size of lysosomes [65,66]. As part of its housekeeping function, starvation-induced unselective macroautophagy also can randomly sequester mitochondria [3,13].

ER-Phagy

The ER is a major center for protein folding and post-translational modifications. A network of quality control systems monitors the fidelity of these processes. During ER-associated degradation (ERAD) misfolded proteins are transported from the ER to the cytosol and degraded by the ubiquitin-proteasome system. ER stress, caused for example by dithiothreitol (DTT), interfering with the formation of disulfide bonds, or the use of tunicamycin, an inhibitor of glycosylation, causes the accumulation of unfolded proteins and induces the unfolded protein response (UPR). The presence of unfolded proteins in the ER-lumen is transmitted to the cytosol by activating the endoribonuclease activity of the transmembrane ER-protein, Ire1. Splicing of the HAC1 mRNA then leads to translation of an active Hac1 transcription factor. Active Hac1 induces the transcription of a large set of genes increasing the ER-folding capacity and stimulating ERAD. To restore ER homeostasis, the autophagic machinery is additionally activated, when either the capacity of the proteasomal system is exceeded or when the misfolded proteins aggregate, impairing their transport to the cytosol [67–69]. Indeed, DTT-treated *S. cerevisiae* cells showed increased Atg8 levels and lipidation [67,69]. Under these conditions the Atg8-dependent formation of autophagosome-like structures containing tightly packed stacks of ER reverses the stress-induced proliferation of ER [67]. Remarkably, the outer membrane of these structures was continuous with the ER and studded with ribosomes, suggesting a role of the ER as a membrane source for at least these specialized type of autophagosomes. The expression of active Hac1 raised the Atg8 levels, but was unable to induce formation of ER-containing autophagosomes. This suggests that induction of ER-Phagy requires also an Ire1 and Hac1-independent signal [67]. Astonishingly, the sequestration, but not the vacuolar degradation of the proliferated ER, seems to be sufficient to restore viability [67]. At the moment it is unclear how ER-membranes are marked for selective uptake into autophagosomes. One possibility is that misfolded proteins or protein aggregates are concentrated in specialized areas of the ER destined for ER-Phagy.

Conclusions

Selective and unselective forms of autophagy play crucial roles in organelle homeostasis. However, many molecular details, for example the recognition of damaged organelles by the autophagic machinery, are hardly understood. Most likely, related mechanisms are used in recognition of intracellularly replicating bacteria [1,2]. We therefore expect that deeper understanding of selective subtypes of autophagy will also provide us with new tools to fight diseases.

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Figure 1. Morphology of macroautophagy and the Cvt pathway in *S. cerevisiae*.

Starvation-induced macroautophagy starts with the formation of a double-layered isolation membrane or phagophore (nucleation). After elongation of this structure, fusion of the edges yields an autophagosome. The fusion of the autophagosomal outer membrane with the vacuole eventually releases an autophagic body into the vacuole for degradation. The morphologically similar Cvt pathway uses smaller transport vesicles, whose biogenesis starts at a cargo complex containing pApeI.

Figure 2. Scheme of the two ubiquitin-like conjugation systems involved in macroautophagy
 After proteolytic processing of its carboxyterminus by the Atg4 protease, Atg8 is coupled to the membrane lipid phosphatidylethanolamine (PE). A second conjugation system attaches Atg12 to Atg5. The Atg12-Atg5 conjugate then forms an oligomer with Atg16 and stimulates the formation of Atg8-PE. As indicated, after elongation of the phagophore the Atg12-Atg5/Atg16 oligomer predominantly localizes to the outer membrane. The release of Atg8 from the mature autophagosome requires Atg4. Green circles: Atg8-PE.

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Figure 3. Macro- and micropexophagy model

A late stage of pexophagy is represented in the figure, which shows the PAS, PC, PVS, VSM, MIPA and pexophagosome, and proteins localized to these structures. The figure summarizes the Atg protein requirements for pexophagy of several yeasts. The diagram depicts a steady state, meaning that not all these proteins are in these structures, and not all of them interact, simultaneously. Almost all the localization studies during pexophagy conditions have been done in methylotrophic yeasts and most of the interaction studies come from *S. cerevisiae*, during growing or starvation conditions. Atg14 has not been found in methylotrophic yeasts and probably is not involved in PtdIns3P formation at the vertex ring of the vacuolar membranes. Dashed arrow indicates speculative trafficking. Ublc: ubiquitin-like conjugation

system, Pik1: PtdIns 4-kinase, PE: Phosphatidylethanolamine, PI3K: PtdIns 3-kinase complex, PI: PtdIns, PI3P: PtdIns3P, PI4P: PtdIns4P.

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Figure 4. Different stages of micronucleophagy

In *S. cerevisiae* the vacuolar membrane and the nuclear envelope/ER form contact sites by the interaction of the ER membrane protein, Nvj1, and vacuolar Vac8 (I). During starvation these junctions bulge into invaginations of the vacuole (II). Then a micronucleus buds off (III) and after fusion of the vacuolar extensions (IV), a vesicle limited by three membranes is released for degradation (V) into the vacuole. Figure adapted from [20].